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26

Root Transformation by *Agrobacterium tumefaciens*

Annette C. Vergunst, Ellen C. de Waal, and Paul J. J. Hooykaas

1. Introduction

Genetic transformation and clonal propagation are techniques that play an important role in the identification and characterization of plant genes and their products. The joint efforts to develop *Arabidopsis thaliana* as a model for genetic and molecular analysis of higher plants have produced methods for in vitro propagation *(1)*, regeneration and transformation *(2–15)* using either *Agrobacterium* or direct gene transfer.

Agrobacterium tumefaciens, which belongs to the family of the Rhizobiaceae, is the etiological agent of the crown gall disease. This plant pathogenic soil bacterium is commonly used for the genetic transformation of plants *(16)*, because of its precise and efficient mode of DNA delivery and its ability to use different tissue types, which may display variation in competence for regeneration and transformation, as targets. *Agrobacterium* will deliver any segment of DNA that is surrounded by two imperfect direct repeats, called border repeats to plant cells. In the natural situation, these border repeats enclose a region of oncogenic DNA, called the T(transferred)-DNA that is present on the Ti(tumor inducing)-plasmid of the bacterium. After its transfer to the plant cell nucleus, the T-DNA is randomly integrated into the plant genome. The binary vector strategy *(17)* was developed to provide a system to easily introduce genes between the border repeats in *Escherichia coli* and use the loaded binary vector in transformation experiments after its introduction into an *Agrobacterium* strain containing a helper plasmid. In this way, any gene of interest linked to a selectable marker gene can be used to produce transgenic plants.

For *Arabidopsis*, several *Agrobacterium*-mediated transfer methods were developed using different ecotypes, bacterial strains, selectable marker genes, and tissue types *(2–5,7–12,14,15)*.

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A frequently followed method is that in which root explants are used to genetically transform *Arabidopsis* ***(15)***. Root cells of *Arabidopsis* are highly competent for regeneration. Sangwan and coworkers ***(18)*** have shown that the competent cells for transformation are present in dedifferentiating pericycle, but only after preculture treatment with phytohormones ***(15)***. Furthermore, root material can easily be obtained in large quantities, which will allow the isolation of many individual transgenic plants. An other advantage of this approach is the possibility to transform *Arabidopsis* roots that are maintained in sustained cultures ***(1)***. These cultures make it also possible to clonally propagate roots from mutants that are affected in plant development or from heterozygous plants. Such roots can then be used directly for transformation, but also for the regeneration of a large number of plants with the desired genetic characteristics. *Arabidopsis* somatic tissues display systemic endopolyploidy ***(19)***. As a consequence of using these somatic tissues for transformation transgenic polyploid offspring can be obtained. The percentage of polyploid transformants seems to be low when root transformation is used compared to leaf and direct gene transfer methods ***(20)***. Different selectable marker genes such as neomycin phosphotransferase *(nptII*, conferring resistance to kanamycin ***[15]***), *bar* (conferring resistance to phosphinothricin ***[21]***), *csr1-1* (encoding an acetolactate synthase resistant to chlorsulfuron ***[22]***), hygromycin phosphotransferase (*hpt* ***[2,23]***), dihydrofolate reductase (*dhfr*, conferring resistance to methotrexate ***[9]***), and negative selective markers such as *codA*, encoding cytosine deaminase ***(24)***, and the HSV thymidine kinase gene ***(25)*** have been described in connection with the root transformation approach.

Our interest in gene targeting, which occurs only at very low frequencies in plants (10^{-4}–10^{-5} ***[26,27]***), made us decide to optimize the transformation efficiency of *Arabidopsis* root explants. The protocol by Valvekens et al. ***(15)*** was modified and adapted to our local conditions. In this chapter, we describe in great detail a highly efficient transformation protocol for ecotype C24 using the *nptII* gene as plant selectable marker. Usually we obtain a transformation frequency of 2–3 putative transgenic calli per root explant (i.e., a 3–5 mm cutting through a single root), although variation between experiments occurs. This frequency is sufficiently high to perform gene targeting experiments. The regeneration efficiency (i.e., the number of calli yielding shoots) reaches 80–95%. Rooting takes place in approx 95% of the shoots. Four to five weeks after the cocultivation period, rooted shoots can be transferred to the greenhouse for seed set, making the overall tissue culture time quite short. Over 95% of the transformants are diploid. Between 40–60% of the primary transformants segregate in a 3:1 (resistant:sensitive) ratio in their offspring, indicating T-DNA insertion(s) at one locus. Of these 3:1 segregating primary transformants, approx 40% contains one insert. The other 60% have T-DNA repeat structures.

For transformation the *Agrobacterium* octopine strain MOG101 ***(28)*** gave optimal results. Both transient and stable transformation frequencies were determined using a binary vector (pPG1 ***[29]***) that harbors chimeric p35S-*gusA* (intron) ***(30)***, pnos-*nptII*, and p35S-*hpt* genes. This chapter describes the successive steps in the transformation protocol. The growth of the donor material, followed by culture of the roots, cocultivation with *Agrobacterium*, the counterselection of bacteria, and the selection and regeneration of transgenic shoots are described. This is followed by rooting of the shoots, seed set, and analysis of transformants The different factors influencing the transformation efficiency, such as growth temperature and light conditions, addition of acetosyringone, and other factors that might help to improve the system will be described. In Section 4., we give additional information on the transformation efficiency of the ecotypes Landsberg *erecta* and Columbia with the described method, selectable markers, and their selection scheme, and additional information of what other researchers have found to be helpful in optimizing the protocol in their hands.

2. Materials

2.1. Tissue Culture Media

Use demineralized (MilliQ) water for preparing media and stock solutions. Stock solutions are filter sterilized through a 0.22-µm filter, except solutions dissolved in dimethyl sulfoxide (DMSO, *see* **Note 1**).

2.1.1. Antibiotic and Acetosyringone Stock Solutions (see **Note 2**)

1. Timentin (ticarcillin/potassium clavanulate, purchased from Duchefa, The Netherlands) is used to counterselect the bacteria after cocultivation. Prepare a stock of 100 mg/mL in H_2O. Filter sterilize and store 1 mL aliquots in Eppendorf tubes at –20°C.
2. Kanamycin (Sigma, St. Louis, MO). Prepare as a 100 mg/mL filter sterilized stock in H_2O. The solution can be used up to several months when stored at 4°C or kept at –20°C for longer time periods.
3. Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenon; purchased from Aldrich Chemie, Germany). Prepare acetosyringone as a 0.2*M* stock in DMSO (*see* **Note 1**). Store at –20°C in 1 mL aliquots

2.1.2. Phytohormone Stock Solutions

1. 2,4-D (2,4-dichlorophenoxyacetic acid): 10 mg/mL in DMSO (*see* **Note 1**)
2. Kinetin (6-furfurylamino purine): 5 mg/mL in DMSO
3. 2-iP (6-(dimethylallylamino)-purine): 20 mg/mL in DMSO.
4. IAA (indole-3-acetic acid): 1 5 mg/mL in DMSO.
5. IBA (indole-3-butyric acid): 1 mg/mL Dissolve powder in a few drops of 0 1*N* KOH Adjust with H_2O. Filter sterilize

Store all stock solutions in small aliquots at –20°C.

The final concentration of the hormones in the media is shown in **Table 1**.

2.1.3. Composition of the Culture Media

The composition of the media is shown in **Table 1**.

1 Prepare liquid growth medium (LGM): dissolve all ingredients (*see* **Table 1**) for LGM, based on B5 macronutrients, micronutrients, and vitamins *(32)*, in 950 mL H_2O Adjust to pH 5.7 with 1*N* KOH. Then adjust the volume to 1 L. Autoclave the medium at 110°C for 20 min (*see* **Note 5**)
2 Prepare callus induction medium (CIM): dissolve all components (*see* **Table 1**), except agar and hormones, in 950 mL H_2O Adjust the pH of the medium to 5 7 and adjust the volume to 1 L. Dispense into bottles, which already contain 0 8% agar (*see* **Note 3**) Add hormones (*see* **Table 1**) and acetosyringone (*see* **Subheading 2.1.1.**) after autoclaving the medium at 110°C for 20 min and cooling down to 60–65°C Make CIM plates both without (for preculture, *see* **Subheading 3.3.1., step 1**) and with acetosyringone (for cocultivation, *see* **Subheading 3.3.3., step 6**) The final concentration of acetosyringone is 20 μ*M*
3 Prepare shoot induction medium (SIM) as indicated for CIM. After sterilization and cooling down of the medium, add the appropriate hormones as indicated in **Table 1** and the antibiotics kanamycin to a final concentration of 50 mg/L and timentin to a concentration of 100 mg/L (*see* **Note 2**).
4. Rooting medium (RM) consists of half strength MS macro *(33)* and B5 micro salts *(32)* Dissolve the components as shown in **Table 1** in 950 mL H_2O Adjust the pH to 5.8 with 1*N* KOH Adjust to 1 L. Dispense into bottles containing 0 7% agar and autoclave at 120°C for 20 min. Prepare plates with 1 mg/L IBA and without IBA (*see* **Subheading 3.3.5.**).
5. Prepare germination medium (GM) (half strength MS salts) in a similar way as RM. Add kanamycin after autoclaving the medium, just prior to pouring the plates, to a final concentration of 40 mg/L.

For preparation of GM-agar plates without IBA 100 × 25 mm Labtek Petri dishes (Nunc) are used. For the other agar plates, Greiner 94 × 16 mm Petri dishes with cams are used. Pouring of the agar plates is performed under sterile conditions in a laminar flow cabinet.

2.2. Seed Sterilization and Growth of Donor Plants

1 Seeds of *Arabidopsis thaliana* C24
2. 1.5-mL Eppendorf tubes
3. 70% ethanol.
4 Micropipet (P1000) and tips.
5 1% hypochlorite solution containing 0.1% Tween-20
6. Demineralized H_2O.
7. LGM in 250-mL Erlenmeyer flasks.

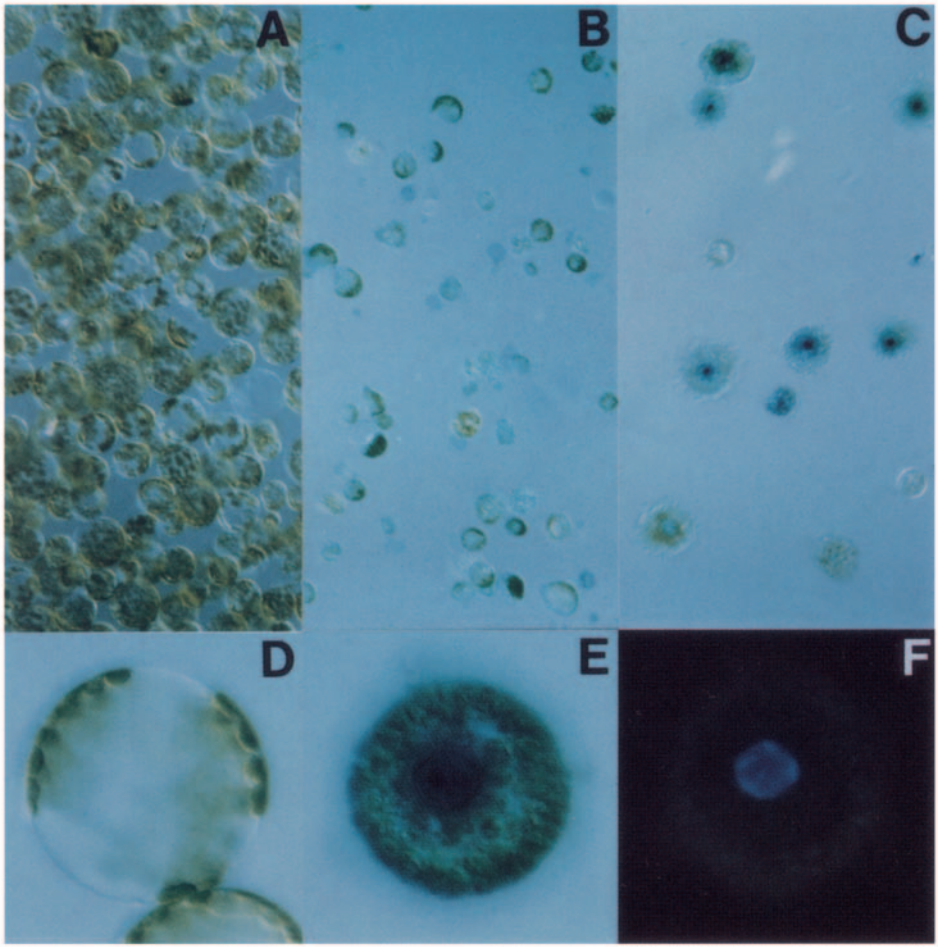

Plate 1 (Fig. 1; see full caption on p. 214 and discussion in Chapter 24). Histochemical localization of GUS activity in *Arabidopsis* mesophyll protoplasts transfected with translational GUS fusions. Purified protoplasts **(A)** were mock-transfected **(D)** or challenged with the following plasmid DNAs: pRTL2-GUS which encodes the nonfused, authentic GUS protein **(B);** pRTL2-GUS-IAA 1 encoding a GUS::IAA 1 nuclear fusion protein (C); pRTL2-GUS-PS-IAA6 encoding a GUS::PS-IAA6 nuclear fusion protein **(E,F).** Transfected protoplasts were cultured for 20 h, stained for GUS activity (B-E) and nuclei (F) as described *(11)*.

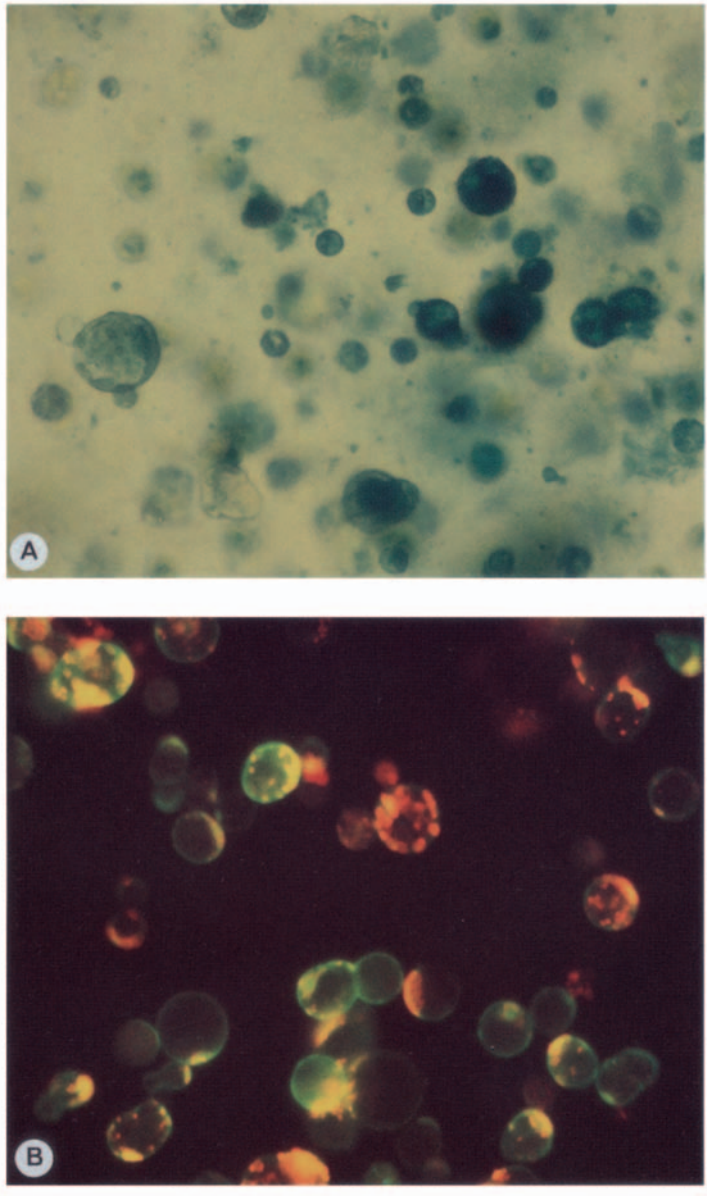

Plate 2 (Fig. 1; *see* full caption on p. 270 and discussion in Chapter 29). GUS and GFP reporter gene expression assays with *Arabidopsis* protoplasts. **(A)** GUS staining of PEG-transformed protoplasts derived from roots of *Arabidopsis* ecotype Columbia after incubation with X-gluc for 6 h at room temperature. **(B)** Leaf mesophyll protoplasts from *Arabidopsis* ecotype Columbia transformed with pCK–GFPs65c exhibit green fluorescence when illuminated with blue light. The chloroplasts emit red fluorescence, whereas the yellow flourescence results from overlapping red and green areas.

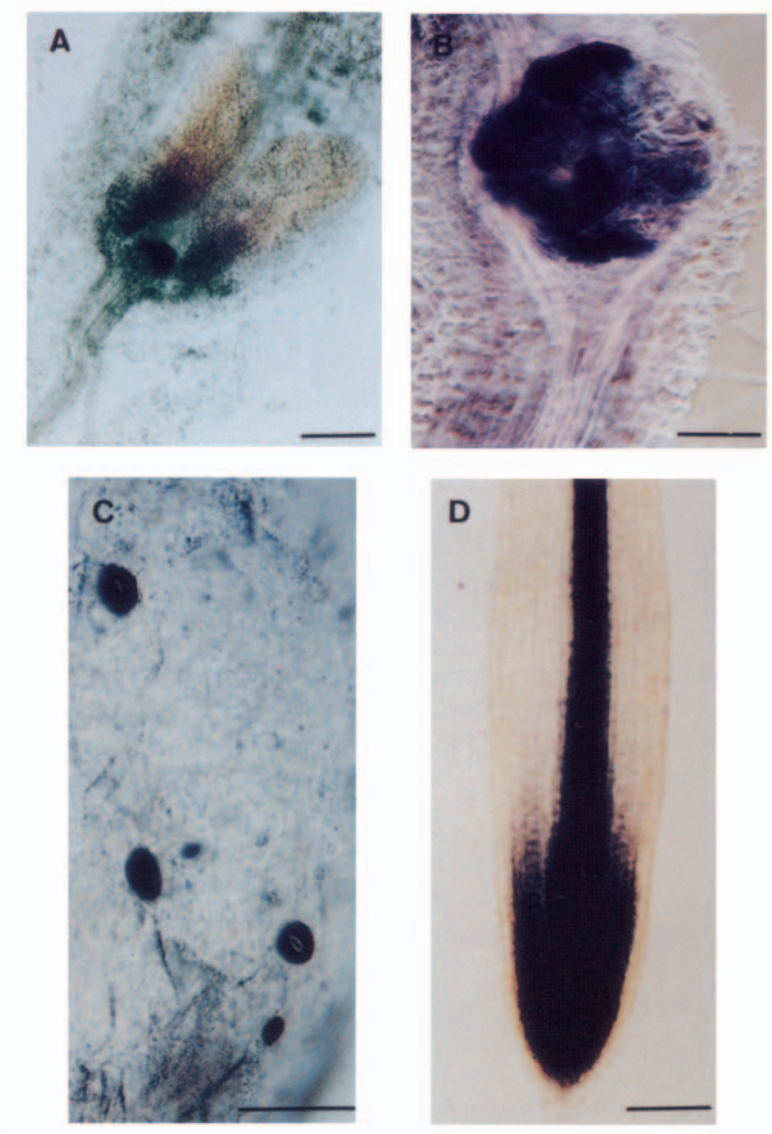

Plate 3 (Fig. 1; *see* full caption on p. 374 and discussion in Chapter 36). (A) WISH on an *Arabidopsis thaliana* seedling hybridized with an antisense *cdc2a* probe detected by gold-labeled antibodies. The signal is visible as a black precipitate resulting from the silver amplification reaction. **(B)** *In situ* localization of *cdc2a* mRNA on a vibroslice of an *Arabidopsis* root infected with a root-knot nematode. The blue precipitate resulted from the histochemical reaction with the substrates X-phosphate and NBT. **(C)** Cotyledon hybridized with an antisense *rha1* probe. Hybrids were visualized by silver amplification of specifically bound gold-labeled antibodies seen as a dark precipitate. **(D)** Chicory root hybridized with an antisense nitrate reductase probe detected by AP-labeled antibodies. A strong expression is visible in the vascular cylinder and in the root meristem. The sample was cleared in CLP for better visualization of the signal.

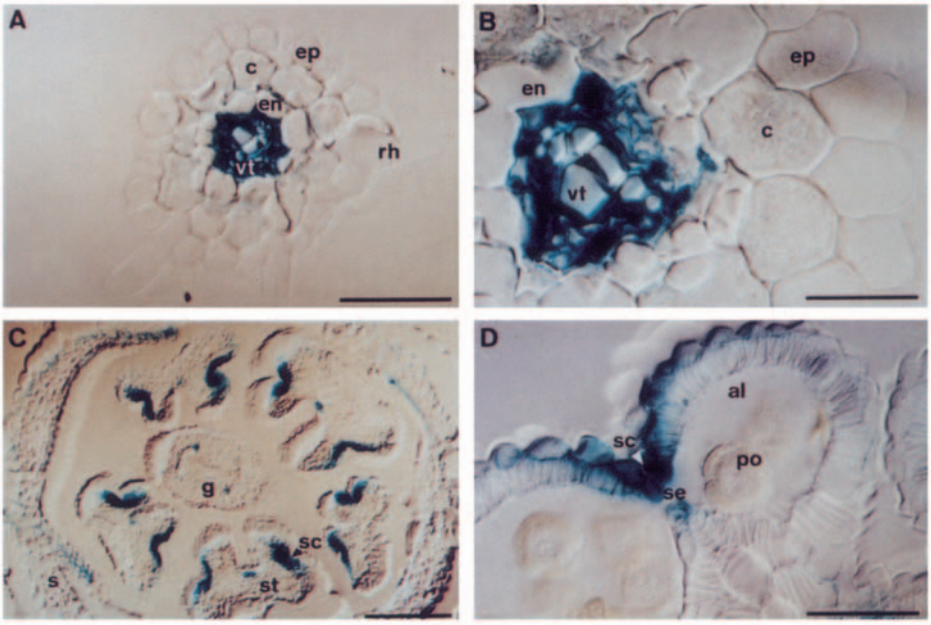

Plate 4 (Fig. 3. *see* full caption on p. 405 and discussion in Chapter 38). X-Gluc reactions on *Arabidopsis* tissue. **(A)** Transverse section through a 2-wk-old root of a *pRPS18A-gus* transformed line *(15)*. **(B)** Detail of the vascular tissue; main GUS activity in vascular tissue. **(C)** Transverse section through a young (stage 9) flower of a line transformed with a *gus* gene fused to a stomium-specific tobacco promoter (provided by T. Beals and P. Sanders, Plant Molecular Biology Laboratory, University of California Los Angeles, CA). **(D)** Detail of mature anther. Main GUS activity located at the stomium, the site of anther dehiscence. Abbreviations: al, anther locule; c, cortex; en, endodermis; ep, epidermis; g: gynoecium; po, pollen; rh, root hair; s, sepal; sc, stomium cells; se: septum; st, stamen; vt vascular tissue. Visualization with Normasky interference microscopy. Bar = 50 μm (A), 20 μm (B,D), and 100 μm (C).

Table 1
Composition of the Root Transformation Culture Media

Component	Concentration				
	LGM	CIM *(15)*	SIM *(15)*	RM *(31)*	GM
Macronutrients[a] (final concentration in mg/L)					
NH_4NO_3				825	825
KNO_3	2500	2500	2500	950	950
$MgSO_4 \cdot 7H_2O$	250	250	250	185	185
KH_2PO_4				85	85
$(NH_4)_2SO_4$	134	134	134		
$NaH_2PO_4H_2O$	150	150	150		
$CaCl_2 \cdot 2H_2O$	150	150	150	220	220
Micronutrients[b] (final concentration in mg/L)					
FeNaEDTA	36.7	36.7	36.7	36.7	36.7
H_3BO_3	3	3	3	3	3.1
$MnSO_4 \cdot 4H_2O$	13.2	13.2	13.2	13.2	11.15
$ZnSO_4 \cdot 7H_2O$	2	2	2	2	4.3
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25	0.125
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025	0.0125
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025	0.0125
KI	0.75	0.75	0.75	0.75	0.415
Vitamins, amino acids, buffers[c] (final concentration in mg/L)					
nicotinic acid	1	1	1		0.5
pyridoxine-HCl	1	1	1		0.5
thiamine-HCl	10	10	10		0.1
glycine					2
myo-inositol	100	100	100		100
MES	500	500	500	1000	500
Carbohydrates (final concentration in g/L)					
glucose	20	20	20		
sucrose				10	20
agar (final concentration in g/L; *see* **Note 3**)					
Daishin agar		8	8	7	7

(continued)

Table 1 *(continued)*

Component	Concentration				
	LGM	CIM *(15)*	SIM *(15)*	RM *(31)*	GM
Phytohormones (final concentration in mg/L; *see* **Subheading 2.1.2.**)					
2,4-D		0.5			
kinetin		0.05			
2-iP			5		
IAA			0 15		
IBA				1	

[a]The macronutrients are made up as 10X concentrated stock solutions We prepare a seperate stock for $CaCl_2 \cdot 2H_2O$ to prevent precipitation (*see* **Note 4**)

[b]Micronutrients are prepared as 1000X stock solutions (*see* **Note 4**) FeNaEDTA (ethylenediamine tetra acetic acid ferric monosodium salt) is made up separately as a 100X concentrated stock solution

[c]Vitamins plus glycine are prepared as a 100X stock Myo-inositol is prepared separately as a 100X stock MES (2-morpholino-ethanesulfonic acid monohydrate) is added as a powder (*see* **Note 4**)

2.3. Transformation Procedure

2.3.1. Preparation and Preculture of Roots

1 10-d-old C24 cultures
2. Empty Petri dishes (Greiner 94 x 16 mm)
3 Scalpels
4 Forceps.
5. CIM plates without acetosyringone
6. Urgopore (Chenove, France) tape

2.3.2 Growth and Preparation of Agrobacterium Strains

1. LC-medium (pH 7 0): Weigh 10 g Bacto-tryptone, 5 g yeast extract, and 8 g NaCl Adjust with H_2O to a volume of 1 L. For agar plates, 1 5% agar (Difco Bacto agar) is added. Sterilize by autoclaving at 120°C for 20 min
2. Antibiotic stocks·
   a Rifampicin (10 mg/mL in methanol, do not sterilize). Final concentration: 20 mg/L.
   b Spectinomycin (125 mg/mL in H_2O, filter sterilized) Final concentration 250 mg/L
   c. Kanamycin (50 mg/mL in H_2O, filter sterilized). Final concentration: 100 mg/L Store the stock solutions at –20°C in 1 mL aliquots
3. Bacteria on a fresh LC-agar plate with antibiotics (*see* **Notes 6** and **7**). *Agrobacterium* stocks are stored at –80°C in LC medium containing 14% glycerol.
4. Liquid LC containing antibiotics.

5. 100 mL flask with cotton wool plug.
6. 28°C growth incubator (shaking)
7. 1 5-mL Eppendorf tubes
8. Micropipet (P1000) and tips.
9. Overnight grown *Agrobacterium* cultures.
10. LGM.
11. Eppendorf centrifuge

2.3.3. Cocultivation of Root Explants with Agrobacterium

1. Empty Petri dishes (Nunc 100 × 25 mm)
2. LGM
3. Bacteria in LGM.
4. Scalpel.
5. Forceps.
6. Filter paper.
7. CIM-agar plates containing 20 μM acetosyringone
8. Urgopore tape.

2.3.4. Selection of Transgenic Calli and Shoots

1. Forceps
2. Sieve (*see* **Note 8**).
3. Empty Petri dishes (Greiner 94 × 16 mm)
4. LGM.
5. Filter paper
6. SIM-agar plates with 50 mg/L kanamycin and 100 mg/L timentin
7. Urgopore tape

2.3.5. Rooting of Regenerated Shoots

1. Forceps.
2. Scalpel.
3. RM-agar plates containing 1 mg/L IBA.
4. Urgopore tape.
5. RM medium without IBA.

2.4. Analysis of Transformants

1. T2 seeds.
2. 70% Ethanol.
3. 1% Hypochlorite solution with 0.1% Tween-20
4. Demineralized H_2O
5. 0.1% Agarose in H_2O
6. Micropipet (P1000) with tips.
7. Solidified GM containing 40 mg/L kanamycin.
8. Parafilm or Urgopore tape.

3. Methods

Perform all steps under sterile conditions in a laminar or down flow (biohazard) cabinet with sterile equipment and sterile instruments. The growth rooms have a 16-h photoperiod and a relative humidity of 50%. The varying light and temperature conditions are mentioned below (*see* **Note 9**).

3.1. Tissue Culture Media

1. After sterilization of the media and cooling down to 60–65°C, add antibiotics and/or hormones (*see* **Subheading 2.1.3.**). Pour 25 mL of agar medium in Petri dishes
2. Allow the agar medium to solidify and dry for 45–60 min with the lids of the Petri dishes slightly opened (*see* **Note 3**) Store the plates at 4°C in a closed plastic bag or wrapped in aluminium foil
3. 50 mL of LGM is dispensed into sterile, 250-mL erlenmeyer flasks capped with aluminium foil

3.2. Seed Sterilization and Growth of Donor Plants

1. Put approx 3 mg C24 seeds in an Eppendorf tube (*see* **Note 10**)
2. Submerge seeds for 1 min in 70% ethanol The seeds will sink to the bottom of the tube when left standing
3. Remove ethanol with a P1000 pipet tip.
4. Add 1 mL of a 1% hypochlorite solution containing 0 1% Tween-20 Make sure all seeds are in contact with the solution Avoid bubbles Let stand for 10 min
5. Remove solution and rinse seeds three times with 1 mL sterile water. Leave small volume of water
6. Add seeds, using the P1000 pipet, to 50 mL LGM in a 250-mL Erlenmeyer flask capped with aluminium
7. Put flask on a rotary shaker (80/100 rpm) in growth room (21°C, 1000–1500 lx, *see* **Notes 9** and **11**)

3.3. Transformation Procedure

For all stages in the protocol, Urgopore gas diffusible medical tape is used (*see* **Note 12**).

3.3.1. Preparation and Preculture of Roots

1. Use roots from 10-d-old cultures (*see* **Note 13**). Separate roots from hypocotyls, cotyledons, and leaves (*see* **Note 14**). Keep the roots in LGM while dissecting them. Place the roots on CIM plates, without drying the roots on filter paper Be careful that all roots are in good contact with the agar-medium. Vegetatively sustained root cultures *(1)* can also be used (*see* **Note 15**)
2. Incubate roots for 3 d in growth room at 25°C with 1000–1500 lx

3.3.2. Growth and Preparation of Agrobacterium Strains

1 Streak bacteria (*see* **Notes 6** and **7**) on a fresh LC-agar plate with rifampicin, spectinomycin, and kanamycin and grow the bacteria for 2–3 d before preparing liquid cultures at 29°C
2. Inoculate *Agrobacterium* strain(s) in 10 mL liquid LC containing antibiotics in a 100-mL flask, plugged with cotton wool, from a fresh LC-plate 1 d before the initiation of cocultivation with root explants. Grow the culture overnight at 29°C in a shaking waterbath or rotary shaker (200 rpm)
3 Measure the OD_{600} of the overnight *Agrobacterium* culture Calculate the desired volume of overnight culture to obtain an OD_{600} of 0 1 in 20 mL LGM and pellet the bacterial cells in an Eppendorf tube (2 min 12,000*g*) Remove the supernatant and resuspend the pellet in 1 mL LGM (Optionally 1 mL of an *Agrobacterium* overnight culture can be used)

3.3 3 Cocultivation of Root Explants with Agrobacterium

1 Transfer roots after the 3-d incubation period to a Petri dish with 19 mL LGM (optional· a sieve can be used, *see* **Note 8**)
2 Add bacteria (from **Subheading 3.3.2., step 3**) The final OD_{600} should be 0 1
3 Incubate bacteria and roots, while shaking from time to time, for 2 min
4 Collect roots and place them in the lid of the Petri dish Cut the roots in pieces of 3–5 mm (one root piece is named explant, *see* **Note 16**). Avoid desiccation of the explants at this stage
5. Dry the root explants on two layers of sterile filter paper (*see* **Note 17**)
6 Place the root explants on CIM-agar plates containing 20 μ*M* acetosyringone (*see* **Note 18**) Make sure the explants are in close contact with the medium
7 Cocultivate the root explants and agrobacteria for 2 d in a growth room (25°C, 1000–1500 lx; *see* **Note 9**)

3.3.4. Selection of Transgenic Calli and Shoots

1. Collect the root explants with forceps (bacteria will have overgrown the explants) and transfer them to a sieve (*see* **Note 8**) that is placed in a Petri dish with LGM. Wash the root explants carefully by shaking the sieve Repeat the washing step in fresh LGM
2. Blot root explants dry on two layers of sterile filter paper (*see* **Note 17**).
3. Transfer the explants to SIM-agar plates with 50 mg/L kanamycin and 100 mg/L timentin (*see* **Note 19**).
4. Transfer the plates to 3000–4000 lx at 25°C (*see* **Note 9**)
5 Transfer the explants to fresh SIM with timentin and kanamycin every 10–14 d for efficient regeneration.
6 Green, putatively transformed calli (usually they start appearing 1–2 wk after cocultivation) are transferred to fresh SIM agar-plates with kanamycin and timentin The calli need subculturing every 2–3 wk (*see* **Note 20**)
7. Shoots will start appearing within 2–3 wk

3.3.5. Rooting of Regenerated Shoots

1. Transfer small shoots to plates with RM containing 1 mg/L IBA. Make sure no callus is left on the shoot (*see* **Note 21**) Put the plates at 21°C with 3000–4000 lx
2. After 1 wk transfer shoots to Petri dishes containing RM medium without IBA Roots will soon develop As soon as a number of roots have grown into the agar, transfer independent transformants (T1) to soil in the greenhouse (*see* **Note 22**)
3. Seeds can be collected 6–8 wk after transfer to the greenhouse
4. Harvest the seeds and store at 4°C (*see* **Note 23**).

3.4. Analysis of Transformants

1. Sterilize about 100 T2 seeds (*see* **Subheading 3.2., steps 2–5**) to test stable Mendelian segregation of the transgene in the progeny
2. Remove water from the last washing step and replace with 400 µL 0 1% agarose, in which the seeds will be evenly distributed
3. Spread seeds in 0.1% agarose on solidified GM containing 40 mg/L kanamycin using a P1000 pipet tip
4. Place dishes for 4 d at 4°C before transfer to the growth room (21°C, 3000–4000 lx, *see* **Note 11**) Sensitivity to kanamycin results in bleached cotyledons and inhibition of leaf and root growth Estimate the ratio of sensitive versus resistant seedlings (*see* **Note 24**).
5. Test the ploidy level of the transformants (*see* **Note 25**), since polyploidy is disadvantageous in further genetic analyses
6. To further analyze the transformants perform Southern blotting or PCR Pool about 20 T2 plants per T1 transformant, grown in soil, to extract chromosomal DNA and estimate the number and mode of T-DNA integration events by Southern hybridization (*see* **Note 26**)

4. Notes

1. DMSO (sterile filtered, purchased from Sigma) and sterile Eppendorf tubes are used to dissolve the hormones, acetosyringone, or antibiotics. After dissolving, do not sterilize Do not flame DMSO! DMSO is toxic, so handle with care and do not breathe fume.
2. Besides using the kanamycin-resistance gene for selection, we also tested the *bar*-gene ***(34)***, *csr1-1* gene ***(22)*** and *hpt*-gene ***(29)*** although we did not optimize the system as for kanamycin (*see* **Note 20**). The *bar*-gene encodes a phosphinothricin acetyltransferase (PAT), that can detoxify phosphinothricin (PPT). The PPT inhibits glutamine synthetase leading to elevated ammonium levels and cell death We use 20 mg/L phosphinothricin (Duchefa, The Netherlands) The stock solution is 50 mg/mL in H_2O, filter sterilized. The transformation efficiency is similar to that when using kanamycin selection. Chlorsulfuron (Glean, DuPont) inhibits acetolactate synthase (ALS). A single base pair mutation (*csr1-1* allele) confers resistance to chlorsulfuron, a sulfonylurea herbicide Selection for the mutated ALS-gene is performed on 5 µg/L chlorsulfuron. The stock is 1 µg/µL in H_2O. The selection is not as efficient as kanamycin selection in our hands, but transgenic plants can be obtained.

Hygromycin (Calbiochem, La Jolla, CA) selection is performed on medium containing 20 mg/L. Hygromycin is very toxic and must be handled with care according to safety standards

Otherwise, we have detected an interaction between agents that are used to inhibit bacterial growth and those for selection of transgenic plant material We previously counterselected bacteria with 500 mg/L carbenicillin (Duchefa, stock 250 mg/mL in H_2O, stored at –20°C) and 100 mg/L vancomycin HCl (Duchefa, stock 100 mg/mL in H_2O stored at 4°C), while selecting for kanamycin-resistant calli with success In later stages, we were not able to use this combination of antibiotics (hardly any calli were obtained), but we found timentin to be a very good alternative for counterselection However, PPT selection is still most efficient in combination with vancomycin and carbenicillin. The apparent transformation frequency is lower in combination with timentin It is known that carbenicillin has auxin-like properties when broken down in vitro *(35)* Because we did not alter the protocol, this sudden decrease in transformation frequency can hardly be assigned to an interaction with the phytohormones in the medium as described recently by Lin et al. *(36)* Furthermore, there seems to be no interaction of vancomycin and/or carbenicillin with PPT selection in our hands The above mentioned results do not imply that these problems will occur in every laboratory or with different ecotypes Unknown factors might have an effect or the production process of the antibiotics might play a role It is clearly meant as a suggestion in case transformation frequencies are unexpectedly low.

The bactericidal compound cefotaxime is thought to decrease the regeneration efficiency as was also found for *Antirrhinum majus (35)*.

3 Agar is purchased from Brunschwig Chemie (Amsterdam, The Netherlands) Often the choice of agar influences the regeneration and transformation efficiency In our hands, Daishin agar gives good results It is very important to take good notice of the way the media are autoclaved. We never autoclave more than 2 L of medium in a pressure cooker at 110°C Extension of the overall sterilization time (e g , by sterilizing more medium) can decrease the consistency of the agar after solidifying. Soft agar makes it very tedious to spread the root explants. The agar should feel quite solid when touching with a tip of a pair of tweezers. Agar plates are dried in a laminar flow cabinet with the lid open for 45–60 min With agar plates prepared in this way, very good regeneration is obtained. Both the consistency of the agar and the lack of accumulation of condensation (by drying the plates and using gas diffusible tape; *see* **Note 12**) yields a high percentage of healthy shoots, without vitrified appearance Furthermore, *Agrobacterium* is counterselected more efficiently on plates that are prepared in this way

The RM and GM medium contain 0.7% agar. The agar is more soft than in CIM and SIM plates to allow root growth in the medium

4 The macro-, micro-, and vitamin stock solutions are not sterilized and are stored at 4°C in the dark. They can be stored up to several months.

5. Media containing glucose are sterilized for 20 min at 110°C.

6 We used *Agrobacterium* strain MOG101 *(28)* for optimization of root explant transformation This strain has a nopaline C58 chromosomal background with a rifampicine-resistance marker and contains an octopine pTiB6-derived helper plasmid. The complete Tr- and Tl-region have been replaced by a spectinomycin-resistance marker The octopine strain LBA4404 *(17)*, the supervirulent strain EHA105 *(28)*, and nopaline strain MOG301 *(28)* yielded somewhat lower transformation frequencies than MOG101

The pBinl9 *(37)* derived binary vector that was used to optimize the transformation protocol (pPG1 *[29]*) has a bacterial kanamycin resistance gene Between the T-DNA borders chimeric pnos-*nptII*, p35S-*gusA* (intron), and p35S-*hpt* genes are present. The bacterial strains and binary vectors used define the antibiotics needed.

7. It is recommended to include controls in each experiment. We use a bacterial strain lacking the *nptII* gene on the T-DNA as a negative control Roots infected with this strain and placed on kanamycin containing medium should not yield transformants. This control allows to test the effectiveness of the selection procedure A few roots infected with a strain harboring the selectable marker gene are grown on medium without kanamycin, but with timentin to test the regeneration efficiency and the medium
8. We use stainless steel sieves with 100 mesh screens (purchased from Sigma) Optionally, sieves can be made by cutting 50-mL Greiner tubes, heating the cut surface, and pressing against a nylon membrane. After contamination with bacteria, the sieves are sterilized at 120°C for 20 min in a beaker together with the liquid LGM from **Subheading 3.3.3., step 3** The sieves are then cleaned with H_2O (do not use soap or any other detergent!), allowed to dry, and sterilized again for further use.
9 We have good results with Philips TL 83HF light tubes which emit light in which the orange/red part of the spectrum is well represented In the different steps of the protocol, the light intensities and growth temperature are varied Growth of the plants for root material takes place for 10 d on a rotary shaker at 21°C with a light intensity of 1000–1500 lx. During and after cocultivation (*see* **Subheading 3.3.3., step 7**) the temperature is increased to 25°C. Comparison of transformation frequencies at 21°C and 25°C revealed a large difference At 21°C the frequency (number of calli per explant) was 6–10-fold lower and the regeneration frequency (number of calli yielding shoots) dropped to 25% When the roots are incubated at 25°C with higher light intensities (3000–4000 lx) after cocultivation (*see* **Subheading 3.3.4., step 4**) the regeneration efficiency reaches frequencies up to 80–95% In case only one incubation temperature is available, a temperature of 25°C is advised, especially during cocultivation.
10 Approximately 3 mg seeds is sufficient for 500–800 explants after cocultivation (*see* **Note 20** for transformation frequencies). Some losses are obtained during the washing steps. It is advisable to initiate more cultures, even if low numbers of transformants are needed, due to possible contamination.
11 Stratification of seeds before transfer to the growth room can be desired Especially when freshly harvested seeds are used, the germination frequency can be low without prior cold treatment. Flasks or agar plates containing the sterilized

seeds are kept at 4°C for 3–4 d The seeds need to be imbibed during the cold treatment Stratification of the seeds synchronizes the germination time Seedlings sown in flasks for transformation can be used 10 d after transfer to the growth room to collect roots (*see* **Subheading 3.3.1., step 1**)

12. Urgopore gas-diffusible tape (Chenove, France) is used at all stages to increase gas exchange. By using parafilm, condensation can accumulate which may cause formation of vitrified regenerants. Furthermore, an increase in concentration of ethylene might negatively influence the transformation and regeneration process Gas diffusible tape may prevent possible negative effects of ethylene action
13 The age of the root material that is used for transformation is of great importance Roots grown for 10 d under our growth conditions yielded significantly higher transformation frequencies than 14- or 21-d-old root material. If other light and/ or temperature conditions are used it could be that the donor plants should be older or younger for optimal transformation efficiency.
14 Forceps with a curved tip (e.g , Medicon 07 72.40, Lamèris, The Netherlands) come in very handy in all steps of the transformation process. After 10 d of growth, a clump of seedlings will have formed Seedlings are easily pulled out in a Petri dish by pulling the leaves with forceps while holding the clump back with a scalpel Then it is easy to cut the roots just below the hypocotyl The roots are kept in LGM Furthermore, we use a black background underneath the Petri dish to make the roots clearly visible
15 Czako et al *(1)* have developed a technique to grow sustained root cultures of *Arabidopsis*, which can also be transformed with *Agrobacterium* This can be especially useful for maintaining lines with a hemi- or heterozygous genotype or with a mutant genotype affecting growth. These cultures can also be used in the herein described transformation protocol Every 2–3 wk the root cultures are treated with 0 05 mg/L IAA for 2 d and transferred to fresh ARC *(1)* medium For transformation with *Agrobacterium,* we treated the root cultures with IAA 10 d before initiating the preculture of the roots (*see* **Subheading 3.3.1.**) and transferred to fresh ARC after 2 d The transformation frequency was about two-fold lower compared to roots collected from 10-d-old seedlings. As the cultures grew older (> 6 mo) the transformation frequency becomes lower and less reproducible in our hands
16. The roots are arranged in a bundle of approx 5 cm in length. Transverse cuttings are made every 2–3 mm Again, the roots are arranged in a bundle and the cuttings are repeated The root explants are further wounded by tapping with a scalpel several times. This will finally result in root explants, which mainly vary in length between 3–5 mm. It is not necessary to wound the roots prior to the 2 min incubation period with bacteria
17 Take about 15–20 explants each time with forceps having a curved point While putting the roots on filter paper, spread the explants directly when still moist. This way the explants will separate easily and can be transferred to a plate containing CIM medium. The explants will stick to the tip of the forceps and can be transferred by tipping them on the agar plate. Doing this, the explants can be easily spread on the agar It is important that the roots are dried, although they

should not dry out! This especially accounts for the transfer to SIM medium (*see* **Subheading 3.3.4., step 3**). If roots are kept too moist it is possible to get *Agrobacterium* overgrowth in later stages Furthermore, regeneration seems to improve when roots are desiccated a little

18 We found that addition of acetosyringone during cocultivation could improve the transformation frequency 1 3–2-fold Sheikholeslam and Weeks ***(14)*** found an increase in transformation frequency using leaf explants. They cultured the agrobacteria with acetosyringone prior to cocultivation, whereas our experience with root explants has shown that addition of acetosyringone during cocultivation is more efficient

19. Good results are obtained if the explants are not touching each other (100–150 per Petri dish). In this way, growth and recovery (and counting, if desired) of independent transformants can be enhanced. When transformation frequencies are within the range of 2–3 calli per root explant, it is difficult to separate independent transformation events It may occur that two or more independently transformed cells give rise to one chimeric callus. Then the number of calli that are counted will even be an underestimation Transient expression studies (performing a histochemical X-Gluc staining 5 d after cocultivation) showed indeed that several transformed cells can be located at the wounded side of the explants For that reason, we continue with one putatively transformed shoot per callus

20 Using the described protocol, we are usually able to obtain a transformation frequency for ecotype C24 of 2–3 kanamycin-resistant calli per root explant Of these calli, approx 80–95% will develop shoots that can be rooted (95%) In the greenhouse between 1 and 5% of the transformants may be lost during adaptation or may be sterile

Besides C24 ,we tested the ecotypes Landsberg *erecta* and Columbia for their transformation efficiency using kanamycin selection For Landsberg *erecta* *Agrobacterium* strain LBA4404 ***(17)*** yielded transformation frequencies similar to C24 transformed with MOG101. The regeneration efficiency of Landsberg (10%) was less efficient under the conditions optimized for C24. Ecotype Columbia gave lower transformation frequencies with MOG101 than C24, although transformed shoots can readily be obtained Transformation of other ecotypes has been described by others, for example ecotype Bensheim and Wassilewskija ***(2)***, Nossen ***(9)*** or RLD ***(25)*** Already a number of optimized root transformation protocols were described. Mandal et al. ***(11)*** for instance described the use of 5-azacytidine to improve the regeneration efficiency. Clarke et al. ***(5)*** mentioned the use of silverthiosulphate, which inhibits ethylene action, to increase transformation efficiencies and the use of an agarose overlay to easily spread the roots The protocol described in this chapter does not require any addition of silverthiosulphate or 5-azacytidine under our local growth conditions Also, the use of an agarose overlay only decreased the transformation frequency In other laboratories though, with differing tissue culture conditions, or different ecotypes or selectable markers used, addition of these compounds might aid in optimizing transformation frequencies. But also under less optimal conditions trans-

formants will be obtained! We encountered severe difficulties with transformation when painting or welding activities were going on elsewhere in the building Especially, when roots during the cocultivation phase came in contact with gasses produced by painting or welding the transformation frequency decreased drastically.

21. The percentage of shoots that will root will be as high as 95%, provided shoots with a healthy appearance are taken. Vitrified shoots develop roots at a lower frequency. Furthermore, make sure to remove all callus. Callus material left on the base of the shoot can inhibit rooting. The shoots are cultured on RM with IBA for 1 wk. Longer incubation on IBA can give the plants a bushy phenotype Rooting is normally performed on medium without antibiotics and/or selective compounds The presence of these compounds can decrease the rooting efficiency *Agrobacterium* growth is very rarely found after transfer of the shoots to RM plates
22. For transfer to the greenhouse (60–70% relative humidity, 19°C, >4000 lx, Philips high pressure lamps HPI-T 400W and SON-T AGRO lamps 400W alternating with Philips 60W light bulbs), plants are used which already developed a few roots. We use normal potting soil, mixed with a little sand, in recycling pots. After transfer to soil, a high humidity is maintained by covering the plants with plastic for at least 1 wk The plants are also covered with filter paper for about 1 wk The plants can adapt to the greenhouse conditions slowly by first making holes in the plastic foil and subsequently removing the foil. Depending on the health of the transformants, the adaptation period may have to be extended Soil is kept humid after transfer of the plants When the plants start bolting, Aracons (Beta Tech, Belgium) are put over the plants This system makes it possible to grow individual transformants separately, avoiding the risk of cross pollination or seed loss *See* Chapter 27 on leaf transformation, **Note 20**, for solving problems with the black fly In vitro seed set can also be obtained in Greiner pots (100–53 mm), but much less seeds are obtained per primary transformant than in vivo in the greenhouse (*see* Chapter 27 on leaf transformation, **Note 19**)
23 Seeds are collected when the siliques have dried Using the Aracon system, the seeds can be easily collected by pulling the plant through the Aracon base.
24. A segregation ratio of 3 resistant to 1 sensitive seedling predicts T-DNA integration at one chromosomal locus (the χ-square test is used to statistically analyze the data). Using the described protocol 40–60% of the transformants segregate 3·1 in their offspring
25 For genetic analysis and the isolation of recessive mutants (e g , by T-DNA tagging approaches), diploid transgenic lines are desired. Polyploidy will hamper further analysis. The level of tetraploid plants in our population of transgenic lines is low Less than 5% of the plants were tetraploid. Tetraploid plants can be recognized by the size of the seeds (larger compared to diploids), leaves, and late flowering phenotype. It is also possible to count chloroplasts in the guard cells of stomatal cells ***(38)***. Therefore, epidermal strips of the leaf can be examined using fluorescence microscopy Tetraploids contain more (> 12) chloroplasts per two guard cells than diploids ***(6–8)***. Altmann et al. ***(20)*** described an easy method to distinguish between diploid and tetraploid plants by measuring the pollen size.

26 A 3.1 segregation pattern in the offspring of a transformant does not necessarily resemble one insert. Often T-DNA repeats are formed By isolating chromosomal DNA of 20 pooled T2 plants and performing Southern analysis we found that approx 40% of the 3:1 segregating transformants indeed contain one insert Less than 1% represents a not transformed plant (=escape).

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